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Art. XXVII.—*Some Observations on the Methods of Using the Agglutination Test in the Diagnosis of Disease in Bovines caused by the Bacillus of Contagious Abortion.*

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1. Materials required on which to found a Diagnosis of Contagious Abortion.

The materials which may be examined are:—

(1) Vaginal or Uterine Exudate after Parturition.

(2) Foetus or Foetal membranes.

(3) Blood serum.

(4) Milk.

In animals which have recently aborted (1) and (2) are applicable, but in animals from which these were not secured, or in others in which it is desirable to diagnose infection in the absence of abortion, or at a time remote from the act of abortion, the most commonly used material is (3) Serum. This is of especial value because of its being easily obtained from cows at all stages of the disease from shortly after infection onwards; also from male animals, proving that they are susceptible to infection, and from laboratory animals, such as guinea-pigs. The drawback is the difficulty of obtaining the serum from a whole herd in the country where dairymen would have to collect the samples.

It was because of this difficulty that investigations have been made as to the possible use of (4) Milk. As far as I have been able to ascertain, this material, on account of its physical properties, has not been used to any extent for the agglutination test. In some respects milk itself is an unsatisfactory material, and for that reason whey, prepared by the artificial clotting of milk, has been used. Although these investigations are incomplete, they furnish evidence strongly suggesting that it will be found satisfactory for diagnosis. Should this be so, milk, from its ease of collection, would be the ideal material for examination in the case of lactating animals.

In this section will be found results of certain researches conducted on diagnosis from specimens of uterine exudate and of whey.

When *whey* has been used the agglutination test alone has been applied to it.

When using exudate[1] *the means available in the hands of the bacteriologist are:—*

(1) Microscopical examination.

(2) Cultures.

(3) Inoculation of animals.

(4) Agglutination test; and

(5) Complement fixation test.

1. *Microscopical examination.*—To a person who is in the habit of frequently examining specimens containing the *bacillus abortus* the diagnosis presents no great difficulties, especially if the organisms are plentiful and typically arranged. On the other hand there are met with in vaginal or uterine material bacteria which simulate more or less the *B. abortus*, and here the other tests aid to confirm the diagnosis.

2. *Cultures.*—For these to be satisfactory, the material should be as free as possible from contamination by other organisms. In Australia a cow is frequently at grass when abortion takes place, and may have aborted some days before the stockman sees her. The discharge in such cases is sure to be badly contaminated.

Also, the discharge should obviously not contain any antiseptic. It sometimes happens that one is called upon to examine material from a cow which has been syringed out with lysol, the specimen smelling strongly of the antiseptic. For these reasons this method is of very limited application.

3. *Inoculation of Animals.*—The remarks made about contamination and antiseptics under the last heading apply equally here. Nevertheless, this method of diagnosis has been used here with success, guinea-pigs being used. Owing to the length of time before marked lesions develop one resorts to the agglutination test of the blood serum of the guinea-pig to determine whether infection by the specific organism has taken place.

4. *Agglutination Test.*—This has proved valuable in diagnosing a sample of exudate, in which the microscopical findings were doubtful, and where, on account of great contamination, cultures were not obtained.

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1 The word exudate is used throughout to mean vaginal discharge containing uterine exudate.

It has been found very delicate, as little as 0.0025 c.c. of exudate causing agglutination. From one of these positive cases the blood serum, secured 16 days later than the exudate and 17 days after abortion, gave an exactly similar agglutinating titre to the exudate. (See Cow II., "Bluey.")

Further, exudate may be kept until it is fairly swarming with bacteria, and yet show little loss of agglutinant action. Also exudate diluted with carbolised saline (10% of exudate in saline containing 0.5% of carbolic acid) shows little loss of agglutinating bodies.

Exudate from a healthy cow which calved at the Institute was tested, and even in an amount of 0.25 c.c., failed to give any agglutination.

5. *Complement Fixation Test.*—No opportunity has presented itself of applying this test to exudate, but, judging from the results with the agglutination test on this material, there seems to be no reason why it should not be of use for diagnostic purposes.[1]

Details of Cows.

1. *Normal Cow.*—This animal was kept at the Institute in connection with another experiment. She calved, after the usual signs of on-coming parturition, the offspring being of full size, and vigorous.

Exudate secured the day she calved gave no agglutination with 0.25 c.c.

Whey from milk taken eleven days after calving gave a positive agglutination with 0.25 c.c., but a negative with 0.05 c.c.

II. Cow, "Bluey."—Detailed history not available. Aborted on 1st September, 1913. *Exudate*—in appearance typical of the disease—was secured from the vagina next day. It was tested four days later, and agglutinated at 0.15 c.c., this being the smallest quantity used. Twelve days later the end-point was determined to be 0.0025 c.c., the material used in this test having been left in the bottle and being now fairly putrid. At the same time material which had been diluted with carbolised saline twelve days ago now gave a reaction with 0.005 c.c.

Microscopical examination of exudate showed long chains of bacilli, streptococci, and also some clumps of bacilli the size of *B.*

1 An opportunity has since presented itself of testing, by the Complement Fixation method, a sample of vaginal exudate from a cow which had recently aborted. The result was positive, thus showing that the test may be also availed of for diagnosis as is suggested above.

typhosus. Smears from placental membrane showed streptobacilli and streptococci, as above. Neither specimen showed bacilli which could be definitely recognised as the bacilli of contagious abortion. *Cultures* of the *B. abortus* were not obtained, the tubes being overgrown by the other organisms present.

III. Cow, "Baptist."—Aborted on 22nd June, 1914. *Exudate*, secured from the vagina two days later, gave agglutination with 0.0025 c.c. *Microscopical examination of exudate* showed numerous organisms morphologically like *B. abortus*.

Inoculation of Animals.—Guinea-pig 488 was inoculated subcutaneously with exudate, and its serum, tested nine days later, gave no agglutination; 40 days after inoculation a positive agglutination was obtained with 0.15 c.c. of serum; 76 days after inoculation 0.0025 c.c. of serum gave a positive agglutination; 95 days after inoculation the animal was killed.

Post-mortem Examination.—Animal in good condition and all organs apparently normal, except the spleen, which showed a nodular appearance externally and on section numerous very small greyish foci, which looked rather more like enlarged and prominent malpighian bodies than foci of necrotic material. On smears, no organisms could be detected.

Guinea-pig 489, inoculated intraperitoneally with exudate, was tested 40 days after inoculation, when the serum gave a positive agglutination reaction.

IV. Cow, "Garfish."—Calved 5th July, 1914, not being due till the 17th of the same month, but the owner remarks: "Calf full size, but dead." *Exudate* was secured two days after parturition, and gave no agglutination when tested, using 0.05 c.c.

Microscopical examination of exudate failed to demonstrate the presence of B. abortus, and cultures gave negative results.

Animals Inoculated.—Guinea-pig 491, inoculated intraperitoneally with exudate, was tested three times (the last test 113 days after inoculation), the serum giving no agglutination. Guinea-pig 492, inoculated subcutaneously with exudate, tested one month after inoculation, gave a similar negative result.

Whey from milk collected eight days after calving, gave a negative result with 0.05 c.c.

2. Technique Employed.

In the following pages details of the technique employed in the use of all the materials examined are given with a view to a

standardisation of the test in the hands of different workers, so that the interpretation and comparison of results may be possible and accurate.

a.—The use of whey and the value of the method.

That the specific agglutinins of *Bacillus abortus* may occur in milk has been mentioned by MacFadyean and Stockman, (1) in the Appendix to Part 1 of the Departmental Committee's Report, p. 28, where they say: "We also found that the milk of an animal which had aborted possessed agglutinating properties up to 1 in 25, but, owing to the opacity caused by the addition of milk to a culture, milk is unsuitable for testing purposes." Whether this product has been used at all in diagnosis I am not aware.

It is obvious that if milk, or milk products, could be used it would be advantageous, owing to the ease of securing specimens; but, as milk, even diluted, is unsatisfactory on account of its physical properties, experiments were made with whey. The whey was obtained by clotting milk with Lactic Acid, the technique being as follows:—

To 9 c.c. of milk, 1 c.c. of a 10% aqueous solution of lactic acid is added and mixed. The coagulated milk is then filtered through either cotton-wool and filter-paper, or filter-paper alone, the latter method being usually applied.

The whey is then diluted, one part to nine parts of carbolised saline (Acid. Carbol. liq. 0.5, Sod. chlor. 0.85, water 100), to form the basal dilution 1 in 10, and incubated over-night. Incubation and subsequent filtration are found necessary, otherwise there may be a deposit of albuminous material, which, though unlike the typical deposit of agglutinated organisms, is not desirable, since it may lead to confusion in reading the results.

With the diluted, incubated, and filtered whey, four tubes, each receiving 0.5 c.c. of standard bacterial emulsion, are put up, containing the following amounts of the basal dilution of whey (1 in 10).

	A.		B.		C.		D.
	1.0	-	0.2	-	0.1	-	0.05 c.c.
(representing	0.1	-	0.02	-	0.01	-	0.005 c.c. of pure whey).

As a control, 1 c.c. of diluted whey is put in a tube without any emulsion.

Carbolised saline is then added till the amount of liquid in each tube is approximately 1.5 c.c.

Thus the series of tubes contain :—

	A.	B.	C.	D.
Whey - -	0.1 -	0.02 -	0.01 -	0.005 c.c.
Emulsion -	0.5 -	0.5 -	0.5 -	0.5 c.c.
Saline - -	0.9 -	0.98 -	0.99 -	0.995 c.c.

The tubes are then shaken and put in the incubator till next day, when the results are read. Further incubation shows little alteration, a tube showing "partial" agglutination—*i.e.*, small deposit, with no "clearing" of the supernatant fluid at the end 18 to 24 hours, may be complete at the end of 36 to 48 hours.

Whey, from milk which had been kept 20 days at room temperature, and clotted naturally, has also been tested, when it was found that this bacterially-produced whey exhibited the same titre as the whole milk.

A large number of experiments with whey have been performed, but as yet no conclusion has been come to as regards the limiting titres upon which a diagnosis may be made. It has been found that the agglutinins in whey increase and decrease in much the same manner as they do in blood serum. The work to date has been chiefly among cows on a property where the disease has been in existence for a number of years, but where the abortions, since the investigations commenced, have been very few—not sufficient to warrant a definite opinion as to the comparative value of whey versus serum. Sufficient has been done, however, to warrant investigation of the value of whey as a material for diagnosis by other workers.

b.—Collection of Serum from Cattle and Guinea-pigs.

Occurrence of the Agglutinins in the Blood of Steers.

Cattle.—Undoubtedly the most satisfactory method of obtaining serum in large quantities is by bleeding from the jugular vein, but as this method takes some time, and often necessitates the casting of the animal, another and simpler method is required where only small quantities of blood, sufficient for diagnostic purposes, are required.

It has been found that such may be best obtained from the small artery which runs with the vein extending along the centre of the dorsum of the ear. This vein is the most prominent and is easily found. The hair is clipped off, and an incision is made across the vein and the artery at a point about mid-way betwen the tip and the base of the ear. The artery may not be cut at the first incision, as

it usually lies underneath the vein, and a second rather free incision may be necessary. From this the blood spurts or drips freely, and 5 to 10 c.c. can be collected in a test-tube. The blood is allowed to clot, the clot loosened from the sides of the tube for about two-thirds of its attachment, and the tube inverted in a conical urine test-glass. After allowing this to stand for some hours, the serum may be collected free from corpuscles.

Guinea-pigs.—The following method has been found satisfactory for obtaining small quantities of serum for diagnostic purposes :—

Centrifuge tubes are put up, containing 2 c.c. of citrated carbol saline (Sod. chlor. 0.85, Sod. cit. 1, Ac. Carb. liq. 0.5, water 100). The margin of the ear of the guinea-pig is then incised with the scissors and held dependant. There is a small artery in this region from which, if it has been cut, the blood drips freely. Six drops of blood are collected in the prepared tubes. If we assume that six drops of blood are equal to 0.3 c.c., then we should have about 0.2 c.c. of serum in each tube, or a dilution of 1 in 10.

The tubes are shaken, centrifugalised, and the supernatant fluid pipetted off and tested in various quantities. Though not quite accurate, this method is sufficiently so for determining whether the animal is harbouring the bacilli, as, for example, those animals inoculated with vaginal exudate or milk from suspected cows. Healthy, non-inoculated guinea-pigs have invariably given a negative reaction, even with 0.1 c.c. of pure serum (*i.e.*, 1 c.c. of the citrated saline mixture), whereas some of our reacting guinea-pigs have given an agglutinating titre of 0.005 c.c., and in one case of 0.0005 c.c.

Examination of the Blood of Steers.

Because of the large number of cows which give a positive agglutination reaction it is important to determine whether agglutination of the Contagious Abortion bacillus is brought about by normal ox serum, and, if at all, to what extent. With a view to obtaining information on this point, experiments have been conducted with the serum of male animals never used for breeding.

The following experiments have been made with the serum of steers. The animals were for human consumption, and the blood was taken, immediately upon slaughter at the abattoirs, into a bottle containing a small quantity of strong (20%) citrate solution. The serum was obtained by centrifugalising and tested as follows :—

Basal dilutions were made containing 1 of serum to 9 of carbolised saline; the amount of standardised emulsion used in each tube was 0.5 c.c. Results:

		0.1	0.06	0.04	0.02	0.01	0.005 c.c. pure Serum.
Steer	1	–	–	–	–	–	–
	2	–	–	–	–	–	–
	3	–	–	–	–	–	
	4	–	–	–	–	–	–
	5	+ +			+ +	+ +	+
	6	–	–	–	–	–	–
	7	+	S	–	–		
	8	–	–	–	–	–	–
	10	S	S	–	–	–	–

+ + Agglutination and clearing.
+ Agglutination.
S Slight agglutination.
– No agglutination.

From the above, it will be seen that, with the exception of Steer 5, no animal gave a positive reaction with less than 0.1 c.c. of pure serum, and only one a definite agglutination with that amount. With regard to the "S" readings, as mentioned elsewhere, we do not count these as positives, as the amount of agglutination is extremely small—only perceptible on very careful naked eye examination.

The serum of the positive steer (5) was also tested by the complement fixation method, and again gave a positive reaction.

(*c*) *Standardisation of the Bacterial Emulsion.*

In the description of the technique adopted by other workers there is a remarkable absence of detail as to the concentration of the bacterial emulsion.

Although Mohler and Traum (2) use a method of standardising bacterial emulsion ("agglutinating fluid"), they do so by comparing it with "the old titred agglutinating fluid," but how this "old agglutinating fluid" itself was standardised they do not say, nor do they indicate what it was like in appearance.

MacFadyean and Stockman (3) prepare "an emulsion of greater turbidity than is ultimately required," and dilute it "until when viewed in one of the small tubes employed for the tests it is faintly hazy in appearance."

In this laboratory a method, based upon comparison of the emulsion to be employed with a suspension of Barium sulphate, has

been used, the idea being suggested by the "Nephelometer" of McFarland (4).

For the test the following solutions are made:—A 1% solution of Barium chloride in distilled water, and a 1% solution of Sulphuric acid in water. Three cubic centimetres of the Barium solution is then mixed with 97 cubic centimeters of the acid solution, shaken, and allowed to stand, to come to a state of chemical equilibrium.

The *B. abortus* is sown on agar in Roux flasks and incubated for two or three days, the water of condensation being run over the surface daily so as to get a good growth over the whole surface. Twenty cubic centimetres of carbolised saline (Ac. Carb. liq. 0.5, Sod. chlor. 0.85, water 100) is then added to the flasks and the growth washed off, shaken thoroughly, and passed twice through filter-paper. Dilutions of this suspension, or "emulsion," as it is generally called, are then made with carbolised saline as follows:—1 c.c. of emulsion and 1 c.c. of carbolised solution; 1 c.c. of emulsion and 2 c.c. of carbolised saline; and so on up to 1 c.c. of emulsion and 10 c.c. of carbol saline. These suspensions are the fluids to be compared with the barium sulphate mixture.

To compare, the Barium sulphate mixture is thoroughly shaken and a small tube of about 1 cm. calibre filled with the fluid, the fluids to be tested being placed in similarly sized tubes and comparisons made over printed paper. This Barium suspension is our standard of opacity for emulsion (Standard X).

Supposing the tube which approximates the opacity of the Barium mixture is that tube which contains 1 c.c. of thick emulsion and 6 c.c. of carbolised saline, then this tube is of the proper standard, and is called "Standard X." The whole of the emulsion may then be diluted down with carbolised saline to the proper strength, or kept as thick emulsion, the standard being now known. In the case instanced, the thick emulsion may be termed "7 X," thereby denoting that it requires diluting to seven times its volume—*i.e.*, adding six times its volume of carbolised saline, to prepare a standardised emulsion "X."

In our tests, in which we make the total volume of fluid in the tube up to 1.5 c.c., we use 0.5 c.c. of this standard emulsion "X" in each tube.

3. Quantitative Factors in the Agglutination Reaction.

(*a*) *Not simply a matter of dilution, but a quantitative reaction.*

An unfortunate terminology has crept into descriptions of agglutination methods—probably a relic from the descriptions of the so-called Widal reactions with the serum of typhoid patients—in which frequent use is made of the term "dilution," to express the amounts of serum (or other diagnostic fluid) necessary to bring about agglutination.

The following experiments show that the sensitiveness of the reaction is to be measured by accurate determination of the minimal quantity of serum employed; in other words, it is not simply a matter of dilution, but a quantitative reaction.

Experiments.—To determine whether—

1. The *relation* of the quantity of pure serum to the quantity of fluid in a tube (*i.e.*, degree of dilution), or
2. The *amount* of pure serum in the tube,

is the determining factor in agglutination of a particular serum.

Serum collected from a cow thirteen days previously was used, a basal dilution of 1 of serum to 49 of carbolised saline being made (1 in 50).

The emulsion was standardised in accordance with the usual method, and found to be of a standard "10 X."

Set Ia.

Ten tubes were put up, as follows:—

	A.	B.	C.	D.	E.	F.	G.	H.	J.	K.
Serum (1 in 50) -	1.0	1.0	1.0	1.0	1.0	0.5	0.5	0.5	0.5	0.25 c.c.
Carbolised saline -	0.0	0.5	1.0	1.5	2.0	1.25	1.5	2.0	2.5	1.75 c.c.
Total volume - -	1.0	1.5	2.0	2.5	3.0	1.75	2.0	2.5	3.0	2.0 c.c.
Relationship of serum to fluid - - 1 in	50	75	100	125	150	175	200	250	300	400

Of each of these dilutions 1 c.c. was put in a tube, and the tubes similarly lettered so that the *amounts* of pure serum in these tubes were:—

A.	B.	C.	D.	E.	F.	G.	H.	J.	K.
0.02	0.012	0.01	0.008	0.006	0.0056	0.005	0.004	0.003	0.0025 c.c.

Emulsion (0.05 c.c., Standard 10 X) was then added to each tube and the tubes incubated till next day, when readings were taken.

Results :—

A, B, and C, agglutination and clearing.

D and E, agglutination.

F, slight agglutination.

G, H, J, and K, no agglutination.

Set Ib.

Another ten tubes were put up similarly to above (Set Ia), but with 0.1 c.c. of emulsion (*i.e.*, double quantity) added.

Results :—

A, agglutination and clearing.

No agglutination in other tubes.

Note :—In these two sets of tubes actual *dilution* of serum in total fluid (neglecting the small amount added with the emulsion) was the outstanding test.

Set IIa.

Serum and carbolised saline were placed in tubes as in Set I. :—

	A.	B.	C.	D.	E.	F.	G.	H.	J.	K.
Serum (1 in 50) -	1.0	1.0	1.0	1.0	1.0	0.5	0.5	0.5	0.5	0.25 c.c.
Carbolised saline -	0.0	0.5	1.0	1.5	2.0	1.25	1.5	2.0	2.5	1.75 c.c.

The bacterial emulsion was added to the whole of the fluid in each tube (not to 1 c.c. of each dilution).

Emulsion added (0.05 c.c.), and tubes incubated as in Set Ia.

Result :—

Agglutination and clearing in all tubes from A to J, inclusive.

No agglutination in K.

Set IIb.

Tubes put up similarly to last set (Set IIa), but with 0.1 c.c. of emulsion (double quantity) added.

Result :—

Agglutination and clearing in A, B, C, D, and E.

No agglutination in other tubes.

The actual *dilution* of the serum in these tubes, therefore, is the same in both tests (Sets I. and II.), but the actual *amount* of serum in each tube is not the same in correspondingly lettered tubes in each test. (See Table I.)

With each set, *control tubes* were put up, containing (1) serum and saline, and (2) emulsion and saline.

Note on Readings.

"Agglutination" is to be interpreted as a definite macroscopic aggregation of organisms into clumps deposited either at bottom of tube or at bottom and along sides of tubes.

"Clearing," where the supernatant fluid is free, to the naked eye, of suspended organisms—*i.e.*, agglutination and sedimentation.

"Slight," where there is a trace of agglutination deposit—not what one might with confidence call a definite agglutination. These "slights" are read as *negative agglutinations*, in considering the agglutination titre of a serum.

TABLE I.

Set I. Tube.	Amount of Serum.	Dilution.	a. Emulsion 0.05 c.c.	b. Emulsion 0.1 c.c.
A	0.02 c.c.	1 in 50	++	+
B	0.012 c.c.	75	++	—
C	0.01 c.c.	100	++	—
D	0.008 c.c.	125	+	—
E	0.006 c.c.	150	+	—
F	0.0056 c.c.	175	S	—
G	0.005 c.c.	200	—	—
H	0.004 c.c.	250	—	—
J	0.003 c.c.	300	—	—
K	0.0025 c.c.	400	—	—

Set II. Tube.	Amount of Serum.	Dilution.	a. Emulsion 0.05 c.c.	b. Emulsion 0.1 c.c.
A	0.02 c.c.	1 in 50	++	++
B	0.02 c.c.	75	++	++
C	0.02 c.c.	100	++	++
D	0.02 c.c.	125	++	++
E	0.02 c.c.	150	++	++
F	0.01 c.c.	175	++	—
G	0.01 c.c.	200	++	—
H	0.01 c.c.	250	++	—
J	0.01 c.c.	300	++	—
K	0.005 c.c	400	—	—

++ Signifies agglutination and clearing.
+ Signifies agglutination.
S Signifies slight agglutination.
— Signifies no agglutination.

Conclusions to be drawn from above.

1.—Using the same quantity of emulsion in each tube, *tubes possessing the same dilution (but different quantities) of serum do not furnish parallel results.* (Compare Set Ia. with Set IIa., Set Ib. and Set IIb., etc.)

2.—Using the same quantity of emulsion in each tube, *tubes containing the same quantity of serum do furnish parallel results.*

3.—The agglutination titre varies with the quantity of emulsion used, for, as is evident in the table (I.)—

(a) With 0.05 c.c. of concentrated emulsion (10 X), the minimum amount of this serum which will produce agglutination is 0.006 c.c.

(b) With 0.1 c.c. of concentrated emulsion (10 X), the minimum quantity of serum required is 0.02 c.c.

Experiments were then carried out to test the effect of dilution on an agglutination system.

Tubes were put up, containing:—

	A.	B.	C.	D.
Pure Serum - -	0.02 -	0.015 -	0.01 -	0.005 c.c.
Emulsion (10 X) -	0.05 -	0.05 -	0.05 -	0.05 c.c.

Test 1.—Volume of fluid in each tube made up with carbolised saline to 1 c.c.

Test 2.—Volume of fluid in each tube made up to 2.5 c.c.

Results, both tests:—

A and B, agglutination and clearing.

C, agglutination.

D, no agglutination.

Test 3.—A tube was put up, containing 0.02 c.c. pure serum and 0.05 c.c. emulsion (10 X), and carbolised saline added *up to 20 c.c.*

Result:—Agglutination.

These tests show that the dominating factor is the *quantity* of serum in each tube, and not the degree of dilution. Of course, if equal quantities of each dilution be taken for testing, the quantity of serum in the tube will vary as the dilution (as is shown in Set Ia.). On the other hand, in the practical application of the agglutination test the important figure is what may be termed the "end-point"—*i.e.*, the smallest quantity of serum which will produce agglutination with a standard quantity of emulsion. The necessity, therefore, arises for taking for all tests an exactly similar

quantity of diluted serum, hence it is advisable to refer to the agglutinating titre of a serum as "so many cubic centimetres of serum," and not as "up to a dilution of such and such." For the statement of the dilution to be of any guide to other workers this must be supplemented by a statement referring not only to the actual amount of diluted serum used, but to the actual quantity of emulsion used also.

Adopting the suggested method—*i.e.*, the statement of the exact quantity of serum used, the amount of emulsion used is the only supplementary factor required to be known, for, as is shown by these experiments, and by the following section, the amount of emulsion markedly and in a regular manner affects the agglutination titre.

b.—Influence of Quantity of Emulsion on Agglutination. Also a note on a peculiarity of agglutination.

As is indicated in the experiments in sub-section (a) of section 3 of this paper, the quantity of emulsion present in a tube in which there is a certain definite amount of serum (or, in other words, the proportion between the amount of emulsion and the amount of serum), has a bearing on whether that particular quantity of serum will show agglutination of the bacilli or not.
whereas with 0.05 c.c. of emulsion (Standard 10 X) agglutination

The tests, Ia. and Ib. (see Conclusion 3, page 382), showed that occurred with quantities of serum varying from 0.006 c.c. up, with 0.1 c.c. of the same emulsion the smallest quantity of serum to give agglutination was 0.02 c.c.

To further elucidate this relationship, a large number of tubes, with varying proportions of emulsion and serum, were put up as indicated in the table (Table 2), in which the results are also shown. On account of the wide range in quantities over which the experiment was made, various concentrations of serum and of emulsion were used in actually making the test.

These basal dilutions were :—

Of Serum :—1 in 5, 1 in 50, and 1 in 500.

Of Emulsion :—A standard suspension "X," and also one standardised to 10 X—*i.e.*, 10 times as strong.

The quantities of emulsion used are stated throughout the table in terms of standard X emulsion, but for those tubes shown in the table as containing 1 c.c. and over of standard X emulsion, the 10 X emulsion was used; the amounts of this (standard 10 X) that

TABLE II.

SERUM. c.c. of pure Serum.	EMULSION. c.c. of Standard X.										
	10	8	6	4	2	1	0.8	0.6	0.4	0.2	0.1
0.2	++	++	++	++	+	+	$+_n$	++	++	++	++
0.16	++	++	++	++	++	+	+	++	++	++	++
0.12	+	+	++	++	++	+	+	+	++	++	++
0.08	—	+	+	++	++	++	$+_n$	+	+	++	++
0.04	—	—	+	+	++	++	++	$+_n$	+	+	++
0.02	—	—	—	—	+	++	++	++	++	++	++
0.016	—	—	—	—	—	+	++	++	++	++	++
0.012	—	—	—	—	—	+	+	++	++	++	++
0.008	—	—	—	—	—	—	+	+	++	++	++
0.004	—	—	—	—	—	—	—	—	+	++	++
0.002	—	—	—	—	—	—	—	—	—	+	++
0.0016	—	—	—	—	—	—	—	—	—	—	—
0.0012	—	—	—	—	—	—	—	—	—	—	—
0.0008	—	—	—	—	—	—	—	—	—	—	—
0.0004	—	—	—	—	—	—	—	—	—	—	—
0.0002	—	—	—	—	—	—	—	—	—	—	—

++ Agglutination and clearing.
$+_n$ Agglutination supernatant fluid nearly cleared.
+ Agglutination supernatant fluid not cleared.
— No agglutination.

were put in being 0.1, 0.2, 0.4, 0.6, 0.8, and 1 c.c. respectively. Similarly in regard to the serum—for those tubes shown as containing 0.04 c.c. of serum and over, the basal dilution of 1 in 5 was used, the quantities of this that were put in being 0.2, 0.4, 0.6, 0.8, and 1 c.c. respectively; for those tubes shown as containing from 0.002 to 0.02 c.c. of serum a basal dilution of 1 in 50 was used, the quantities being 0.1, 0.2, 0.6, 0.8, and 1 c.c. respectively; for those tubes shown as containing less than 0.002 c.c. of serum, a basal dilution of 1 in 500 was used, the quantities of this that were put in being 0.8, 0.6, 0.4, 0.2, and 0.1 c.c. respectively.

In each tube the total quantity of fluid was made up to (approximately) 2 c.c. Control tubes were put up, (1) of serum, and (2) of emulsion, and in each case remained unchanged.

These tests show that the quantities of emulsion and of serum combining to produce agglutination bear a direct relationship to one another. The result is particularly striking if one takes the extreme results ("the agglutination and clearing," end-point, shown by + +), which are found to form a straight line when plotted as a graph.

It will also be noted that this arrangement is kept up over the whole length of the series.

One further point is evident from this table (Table 2)—namely, that there is a partial inhibition with certain proportions of emulsion and serum, as may be seen from looking at the tubes containing :—

2 c.c. emulsion and 0.2 c.c. serum;

1 c.c. emulsion, and 0.2, 0.16, and 0.12 c.c. serum;

0.8 c.c. emulsion, and 0.2, 0.16, 0.12, and 0.08 c.c. serum;

0.6 c.c. emulsion, and 0.12, 0.08, and 0.04 c.c. serum;

0.4 c.c. emulsion, and 0.08 and 0.04 c.c. serum;

And 0.2 c.c. emulsion, and 0.04 c.c. serum.[1]

Comparing these with tubes lower down in the same column where the amount of serum is less, we find that where the smaller quantities of serum are used there is again complete agglutination and clearing. Further, where larger quantities of serum are used, there is also agglutination and clearing, the inhibition thus being apparently zonary. This is well seen in the columns of tubes containing—0.8, 0.6, 0.4, and 0.2 of emulsion.

Further, in the table, this zone of inhibition is found to lie approximately parallel to the agglutination end-point; it seems,

1 An enclosing line is used in the table to make these tubes more evident.

therefore, as if there were at least two maxima of agglutination, for a given quantity of serum, varying with the quantity of emulsion, and, between these two maxima, the zone of inhibition.

A peculiar agglutination phenomenon similar to this had been noted before with serum from the same animal.

In this previous test the same amount of emulsion was used in each tube, and the following quantities of serum was placed in tubes :—

Tube.	A.	B.	C.	D.	E.	F.	G.
	0.15 -	0.1 -	0.075 -	0.05 -	0.02 -	0.01 -	0.005 c.c.
Result -	+ -	+ -	+ -	++ -	++ -	+ -	–

After incubation for 24 hours, there was agglutination deposit in all the tubes except G, but there was a marked increase of opacity of the supernatant fluid going from C. to A—*i.e.*, with the greater amount of serum. The only tubes where the supernatant fluid cleared were D and E. After incubating for a total of four days, all the tubes—A, B and C—showed clearing. Emulsion controls, it should be noted, remained unchanged—*i.e.*, were not sedimented. It was considered at the time that, as the most outstanding feature was the failure of A, B and C to sediment, the cause might be physical, and that the reason sedimentation did not occur was because of the increased viscosity in these tubes, due to the large amount of serum. In view of the further experiment detailed above in Table 2, and of the mention by Hewlett of a similar phenomenon of a zone of inhibition with *M. Melitensis*, no suggestion as to the cause is offered. No opportunity of consulting the work referred to by Hewlett has been possible, but the phenomenon, in the main, seems parallel.

The phenomenon is of importance in that an apparent falling off in the agglutinating power of a serum does not necessarily mean that the end-point is to be expected in the next tube.

There may be a zone of lessened agglutination, and then a further increase may be met before the end-point of agglutination reaction. This "end-point" of reaction figure is important in Contagious Abortion, as it affords a means of comparing an animal's condition from time to time as regards the progress of the disease.

c.—Optimum Amount of Emulsion to Use.

Having determined the points referred to earlier in this paper, the question of optimum quantity of bacterial emulsion naturally arises.

Here, again, there being no universal standard adopted, observers cannot strictly compare their results. Thus, to say that an animal, 0.05 c.c. of whose serum produces agglutination, should be considered as affected, in reality conveys no definite meaning, in view of the experiments in sub-sections (a) and (b) above, unless the amount of emulsion be stated at the same time. On the other hand, workers find by experience what is a convenient quantity of emulsion to use, based on the size of the tubes employed, etc., and having found this amount retain it as a standard and use this in future; their own results, therefore, are strictly comparable with one another, but not with those of other workers.

The following experiments were made to determine what quantity, allowing for ease of reading after 24 hours' incubation, was suitable to use.

Material.

Serum, from Cow (as used in previous tests).

Emulsion (standardised, = "10 X").

Four sets of tubes, numbered 1, 2, 3, 4, were put up, using a different quantity of serum in each set. Each set consisted of four tubes—A, B, C, and D, and the quantity of emulsion used was :—

in the A tubes	-	0.05 c.c.	(10 X emulsions)
„ „ B „	-	0.025 c.c.	„ „
„ „ C „	-	0.01 c.c.	„ „
„ „ D „	-	0.005 c.c.	„ „

To Set I. was added 1 c.c. diluted serum (equal to 0.02 c.c. pure serum), and carbolised saline was added, to make the Total Vol. 2 c.c.

To Set II. was added 0.5 c.c. diluted serum (equal to 0.01 c.c. pure serum), and carbolised saline was added, to make the Total Vol. 2 c.c.

To Set III. was added 0.25 c.c. diluted serum (equal to 0.005 c.c. pure serum), and carbolised saline was added, to make the Total Vol. 2 c.c.

To Set IV. was added 1 c.c. diluted serum (equal to 0.02 c.c. of pure serum), and carbolised saline was added, to make the Total Vol. 20 c.c.

Controls.—Serum controls were put up, and remained unchanged.

Emulsion control tubes, of each quantity of emulsion used, with carbolised saline added, were put up, and remained unchanged.

Sets I., II., and III. were read at the end of 24 hours' incubation, the result being :—

	Emulsion.							
	A		B		C		D	
	0.05		0.025		0.01		0.005 c.c.	
Set I. (containing pure serum 0.02 c.c.) -	+	-	+	-	+	-	+	
Set II. (containing pure serum 0.01 c.c.) -	+	-	+	-	+	-	+	
Set III. (containing pure serum 0.005 c.c.) -	+	-	+	-	+	-	+	
Set IV. (containing pure serum 0.02 c.c.) -	+	-	+	-	+	-	?	

Note:—Set IV., at the end of 24 hours' incubation, showed positive agglutination with 0.05 c.c. of emulsion—*i.e.*, in Tube A, but not in the other tubes—B, C, and D.

In tubes B and C, containing 0.025 and 0.01 c.c. emulsion, a positive agglutination was manifest at the end of three days' incubation, but the deposit in tube D, with 0.005 c.c. emulsion, even at the end of this time, was very small indeed—in fact, barely appreciable.

The controls, it should be mentioned, remained unchanged.

From these experiments it appears as if the large volume of fluid (20 c.c.) in Set IV. affected the rate of sedimentation of the clumps of bacilli in those tubes with the smaller quantities of emulsion. As, however, such a large quantity of fluid is not used in the tubes in making a diagnostic test, these results in Set. IV. are not of great importance. In the practical application of the test the total volume of fluid in each tube is usually made up to about 1.5 c.c. A number of tests have also been made in which the total amount was 2 c.c. The tubes in Sets 1, 2 and 3, where the total volume has been made up to 2 c.c., are, therefore, of primary importance. Here it is found that such a quantity of emulsion and of total fluid have been employed that the complete agglutination reaction takes place within the first 24 hours.

Tubes of 2.5 c.c. capacity have been found very suitable in making the test, and the amounts of diagnostic material (serum, whey, etc.), and of emulsion, are, even over a large range of quantities, but involving only a few different basal dilutions, easily contained in 1.5 or 2 c.c. of fluid. Also, 24 hours is a convenient time for incubation before reading the results.

Where the total volume of fluid in each tube is made up to 1.5 or 2 c.c., with incubation extending over 18 to 24 hours, *the optimum of emulsion will be the smallest quantity which gives results that are easily read*, whether the agglutination reaction be positive or negative.

The deposit following agglutination should be such that it can be easily distinguished by the naked eye, even though, as is frequently the case, the whole of the organisms be not sedimented.

In the absence of deposit—*i.e.*, in a negative agglutination reaction, there should be such a quantity of emulsion that a tube containing it can be easily distinguished from a tube not containing any emulsion.

It is of interest here to note the naked eye appearance of the emulsion controls to the sets of tubes under review.

In four of these controls the total volume of fluid was made up to 2 c.c., with carbolised saline; in the other four to 20 c.c.

Amount of Emulsion.	Total Volume.	Naked Eye appearance.
(1) 0.05 c.c. -	2 c.c. -	cloudy.
(2) 0.025 c.c. -	2 c.c. -	faint cloudiness.
(3) 0.01 c.c. -	2 c.c. -	trace of cloudiness.
(4) 0.005 c.c. -	2 c.c. -	no cloudiness.
(5) 0.05 c.c. -	20 c.c. -	faintly hazy.
(6) 0.025 c.c. -	20 c.c. -	trace of haziness.
(7) 0.01 c.c. -	20 c.c. -	haziness appreciable only on comparison.
(8) 0.005 c.c. -	20 c.c. -	no haziness.

Of those emulsion controls containing 2 c.c. of total fluid, it will be seen that (1) and (2) above possess such a degree of cloudiness that they are readily distinguishable to the naked eye as containing emulsion.

These tubes contain 0.05 c.c. and 0.025 c.c. respectively.

In Sets I., II., and III., of agglutination results recorded above the smallest deposit (positive agglutination), which is easily read (tubes containing 2 c.c. of fluid), is that where there is 0.025 c.c. of emulsion.

In Set IV., the only tube where (although there was the same quantity of serum in each tube), agglutination was manifest in 24 hours, was that one in which there was 0.05 c.c. of emulsion in the tube.

With this large volume of fluid (20 c.c.), no smaller quantity gave a completed reaction in 24 hours.

From these experiments, therefore, it has been concluded that the optimum amount of emulsion to use is 0.05 c.c. of "Standard 10 X" emulsion (or 0.5 c.c. of "Standard X" emulsion).

This amount, 0.5 c.c. of "Standard X" emulsion has, therefore, been adopted for use in all practical diagnostic tests for the reasons that—

(1) It gives a marked naked eye deposit (and hence is easily read), in a positive reaction;

(2) Conversely, it gives a definitely cloudy appearance (and hence is easily read), in a tube where there is no agglutination.

(3) With the total volume of fluid in the tube anything from $1\frac{1}{2}$ to 20 c.c., the agglutination reaction is complete in 24 hours.

(4) It is the minimum amount of emulsion that will answer the above requirements.

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